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**13. ABSTRACT** ***(Maximum 200 Words)***

This project was designed to study the incidence and significance of caspase deficiency in breast cancer carcinogenesis and resistance. Task 1 for the first 15 months was to detect caspase expression in breast cancer cell lines and breast cancer tissues. In the past 12 months, we have expanded and cryopreserved 20 cell lines for future study. Detection of caspase expression from breast cancer cell lines revealed that caspase levels varied significantly amongst different cell lines. For example, caspase 6, 8 and 10 levels in HS-578T cells were significantly lower than that of other cell lines. Detection of caspase 3 expression in 40 breast cancer tissues using either immunohistochemistry or Western blot indicated that approximately one fifth of breast cancer tissues had abnormally low or deficient caspase expression. These data suggests that caspase down-regulation/deficiency may have clinical significance, which is consistent with our hypothesis. Caspase detection/screening is currently being continued. Another part of our work was on the characterization of caspase 3 reconstituted MCF-7 cells in response to chemo- and radio-therapies. We found that caspase 3 reconstitution sensitizes MCF-7 cells to both chemo- and radio- therapies. The results have been presented at meeting and a manuscript has been submitted to Cancer Research (under revision).

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## Introduction

Apoptosis (programmed cell death) is a fundamental process involved in homeostasis and the biochemical response to anti-tumor therapy. Aberrant expression of apoptotic regulators, such as Bax-a and p53, has been associated with breast carcinogenesis and therapeutic resistance. Apoptosis is mediated by caspases, a group of proteases. We and others have shown that caspase 3, an effector caspase, is deficient in MCF-7 breast cancer cells. This project was designed to study the incidence and significance of caspase deficiency in breast cancers. The three specific aims were:

1. To determine the incidence and pattern of caspase deficiencies in breast cancer via screening for specific caspase expression in breast cancer derived cell lines, explant cultures and snap frozen human breast cancer tissue.
2. To correlate caspase data (obtained in aim 1) with apoptosis induction using immune modulators (TNF-□, anti-Fas, GrB/Ad), chemotherapeutic agents and radiation on breast cancer derived cell lines and explant cultures.
3. To define the biologic role of specific caspase deficiencies via reconstitution of deficient caspases and comparative studies to define apoptosis induction in response to immune modulators, chemo- and radiation therapy.

## Body

A. According to the original Statement of Work, the task for the first 15 months (task 1) was to screen caspase expression in commonly used breast cancer cell lines and breast cancer derived explant culture. We have made the following accomplishments of this task in the first 12 months.

1). We have expanded 20 breast cancer cell lines (Table 1, Appendix A). Each line was expanded in an optimized medium and cryopreserved in multiple vials for future study (Task 1, a).

2). We have optimized anti-caspase reagent/conditions for immunohistochemical assays, obviating the need to utilize frozen samples for expression studies (Task 1, b).

3). We have made lysates from all 20 cell lines listed above and from 12 snap frozen breast cancer tissues (Task 1, c).

4). We have detected the protein levels of caspases 3, 6, 7, 8, 9 and 10 from the lysate made from above cells lines (Part of Task 1, e). Fig. 1 of Appendix B shows the expression profile of 6 caspases in 9 breast cancer cell lines. The results indicate that the caspase levels varied significantly amongst different cell lines. Caspases 3, 6 8 and 10 had more obvious fluctuation. HS-578T and MDA-MB-157 showed lower expression levels of several caspases (i.e., caspases 3, 6, 8 and 10). In contrast, MDA-MD-436 showed higher expression levels of these caspases. These data provided the basis for further functional screening as proposed in task 2. As the screening continues, we expect to find more cell lines with variable caspase expression.

5). We have detected caspase 3 levels from 9 snap frozen breast cancer tissues. As shown in Fig. 2, Appendix B, caspase 3 levels in samples No. 2, 7 and 9 were relatively lower than that of the other samples. The cause of the down-regulated caspase 3 levels in these cells needs further investigation. Since breast cancer tissue contains multiple cell types, the histology data of these samples will be used as the reference (Task 1, b and e).

6). To obtain more breast cancer cells, we have prepared primary explant cultures from fresh surgically removed breast cancer tissues. Although we have not obtained a stable cell line due to the difficulty of breast cancer tissue culture *in vitro*, we have established digestion, filtration and plating conditions for primary explant tissue culture (To facilitate Task 1, d).

7). We have evaluated caspase 3 protein levels in 30 paraffin-embedded breast cancer specimens using immunohistochemical techniques. With parental MCF-7 cells as the negative control and MCF-7/C3 (MCF-7 reconstituted with caspase 3) as the positive control (Fig. 3A and 3B, Appendix B), we have found that caspase 3 levels varied amongst different breast cancer tissues. Five out of 30 (17%) cases showed loss of caspase 3 expression. Twenty-five (83%) showed normal caspase 3 expression. (Fig. 3C and 3D in Appendix B represent caspase 3 positive and negative breast cancer tissues, respectively). This is consistent with our hypothesis and is encouraging preliminary data (Extension of Task 1, b).

B. Another major part of our effort in the first 12 months was on Task 4, the characterization of caspase 3 deficiency as a contributing factor in breast cancer carcinogenesis and therapeutic resistance. Although proposed for later study in original Statement of Work, we did not delay these experiments because of its translational importance of these studies. Results from these experiments have facilitated meeting presentations and a journal manuscript (Appendices C, D and E) (Task 4).

1). As detailed in Appendices C and D, we have characterized the apoptotic responses of MCF-7 cells reconstituted with caspase 3 and correlated caspase 3 deficiency with chemotherapeutic resistance. Caspase 3 reconstitution significantly sensitized MCF-7 cells to doxorubicin and other chemotherapeutic agents. Our data suggested that caspase 3 deficiency might contribute to MCF-7 resistance to chemotherapy. We also found that activation of caspases 6 and 7 in the drug treated cells were largely dependent on caspase 3 activation. These data provide novel hypothesis regarding signal amplification within apoptotic pathways. Our results were presented at the 22$^{nd}$ San Antonio Breast Cancer Symposium and a manuscript has been submitted to Cancer Research (under revision).

2). As detailed in Appendix E, we have studied apoptosis in caspase 3 reconstituted MCF-7 cells in response to radiotherapy. Caspases 3 reconstitution significantly enhanced the efficacy of radiotherapy in this model system. Although it is believed that both chemotherapy and radiotherapy induce apoptosis through a similar DNA damage pathway, we have found that the detailed biochemical reaction may have subtle

differences when induced by two therapies. A notable finding was that, after adjustment using other cellular apoptotic substrates (i.e., PARP and Lamin B), caspase 3 mediated actin cleavage is relatively more sensitive to radiotherapy than that to chemotherapy. These results were presented at 91st AACR meeting.

**Key Research Accomplishments**

- We have expanded and cryopreserved 20 breast cancer cell lines
- We have made lysate from these cell lines for Western Blot
- We have detected caspases 2, 3, 6, 7, 8, and 10 in 9 breast cancer cell lines and caspase 3 in 9 breast cancer tissues using Western Blot.
- We have detected caspase 3 by immunohistochemistry in 30 cases of breast cancer and found that17% expressed abnormally low/non-detectable caspase 3.
- We have found that reconstitution with caspase 3 sensitized MCF-7 breast cancer cells to chemotherapy induced apoptosis. The results were presented at the 22nd San Antonio Breast Cancer Symposium (1999) and the manuscript is under revision for Cancer Research.
- We have shown that caspase 3 reconstitution enhances the efficacy of radiotherapy induced apoptosis in MCF-7 cells. These results were presented at the 91st AACR meeting (San Francisco, 2000).

**Reportable Outcomes**

1. Manuscript: Reconstitution of caspase 3 sensitizes MCF-7 breast cancer cells to doxorubicin induced apoptosis, Cancer Research, submitted (Appendix C)
2. Abstract: Reconstitution of caspase 3 sensitizes MCF-7 breast cancer cells to doxorubicin induced apoptosis, presented at the 22nd San Antonio Breast Cancer Symposium (Appendix D)
3. Abstract: Reconstitution of caspase 3 sensitizes MCF-7 breast cancer cells to radiation induced apoptosis, presented at the 91st AACR meeting (Appendix E)

**Conclusion**

Summary

In the past 12 months, we have pursued this project in two areas. One was the characterization of caspase 3 reconstituted MCF-7 cell in response to chemotherapeutic agents (doxorubicin and etoposide) and radiotherapy. Our results indicated that caspase 3 reconstitution sensitized MCF-7 breast cancer cells to chemo- and radiotherapy. It strongly suggested that caspase 3 deficiency in MCF-7 cells might contribute to their carcinogenesis and therapeutic resistance, which supported our hypothesis. The other area of emphasis was the systematic screening of caspase levels in breast cancer cell lines and breast cancer specimens. Although the screening is still in progress, we have detected approximately one fifth of abnormally low/non-detectable expression of caspase 3 in breast cancers either by immunohistochemistry or Western blot. This suggests that down-regulation or deficiency of caspase 3 may have clinical significance. Screening of other

caspases will be continued similar to our work on caspase 3. Intensive screening is currently being performed in our laboratory.

"So what section"

Because we began with the characterization of caspase 3 reconstituted MCF-7 cells, experiments in Task 1 were slightly delayed. However, since the experimental conditions have now been established, screening for other caspases as proposed will be a rapid process. Based on our recent experience with primary cell cultures, we have also found that breast cancer explants were difficult to expand. As a result we will focus on the utilization of human breast cancer tissues. The results from our preliminary immunohistochemical experiment are exciting. We will increase, therefore, caspase screening on breast cancer tissues using immunohistochemistry and Western blots.

In the original proposal, Northern blot screening of each specimen was planned. That was proposed because good antibodies were not available for each caspase. Since good antibodies against most caspases are now commercially available (for immunohistochemistry and Western blot), we will now perform Northern blot detection only on those specimens with very low or non-detectable levels of caspase proteins. We will focus on the cases with abnormal caspase protein levels to determine RNA or DNA defects are identified. We will use laser capture microscopy techniques to isolate caspase negative breast cancer cells from stromal elements. RNA will be made from isolated cells, followed by RT-PCR detection of mRNA of certain caspase. These minor changes will make our study more informative. All other experiments will be carried out as planned.

**References**

No direct reference is listed in this section. Appendix C has a list of related references.

**Appendices**

A: Collected breast cancer cell lines and the culture conditions
B: Detection of caspase expression in breast cancer tissues and cell lines
C: Manuscript: Reconstitution of caspase 3 sensitizes MCF-7 breast cancer cells to doxorubicin induced apoptosis
D: Abstract: Reconstitution of caspase 3 sensitizes MCF-7 breast cancer cells to chemotherapeutic agent induced apoptosis, 22$^{nd}$ San Antonio Breast Cancer Symposium, 1999
E: Abstract: Reconstitution of caspase 3 sensitizes MCF-7 breast cancer cells to radiation induced apoptosis, 91$^{st}$ AACR Annual Meeting, 2000

Appendix A: Collected breast cancer cell lines and the culture conditions

| Cell lines | | Culture Conditions | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Number | Name | Medium | Serum | L-gultamine | Na pyruvate | NEAA* | NaHC03 | Insulin | Glucose | EGF |
| 1 | BT-20 | EMEM | 10% | 2 mM | 1 mM | 0.1 mM | 1.5 g/L | | | |
| 2 | BT-474 | 1640 | 10% | 2 mM | 1 mM | | 1.5 g/L | 10ug/ml | 4.5g/L | |
| 3 | MDA-MB-134-VI | L15 | 20% | | | | | | | |
| 4 | MDA-MB-157 | L15 | 10% | | | | | | | |
| 5 | MDA-MB-231 | L15 | 10% | | | | | | | |
| 6 | MDA-MB-361 | L15 | 10% | | | | | | | |
| 7 | BT-483 | 1640 | 20% | 2 mM | 1 mM | | 1.5 g/L | 10ug/ml | 4.5g/L | |
| 8 | Hs 578T | DMEM | 10% | 4 mM | | | 1.5 g/L | 10ug.ml | 4.5g/L | |
| 9 | MDA-MB-330 | L15 | 20% | | | | | 16ug/ml | | 30ng/ml |
| 10 | MDA-MB-415 | L15 | 15% | | | | | 10ug/ml | | |
| 11 | MDA-MB-435S | L15 | 15% | | | | | 10ug/ml | | |
| 12 | MDA-MB-436 | L15 | 10% | | | | | 10ug/ml | | |
| 13 | MDA-MB-453 | L15 | 10% | | | | | | | |
| 14 | MDA-MB-468 | L15 | 10% | 2 mM | | | | | | |
| 15 | T47D | 1640 | 10% | 2 mM | 1mM | | 1.5 g/L | 10ug/ml | 4.5g/L | |
| 16 | ZR-75-1 | 1640 | 10% | 2 mM | 1 mM | | 1.5 g/L | | 4.5g/L | |
| 17 | ZR-75-30 | 1640 | 10% | | 1 mM | | | | | |
| 18 | SK-BR3 | McCoy's 5A | 10% | | | | | | | |
| 19 | MCF-10A | MEGM | 10% | 2 mM | | | | 10ug/ml | | 30ng/ml |
| 20 | MCF-7 | 1640 | 10% | | 1 mM | | 1.5 g/L | | | |
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Appendix B

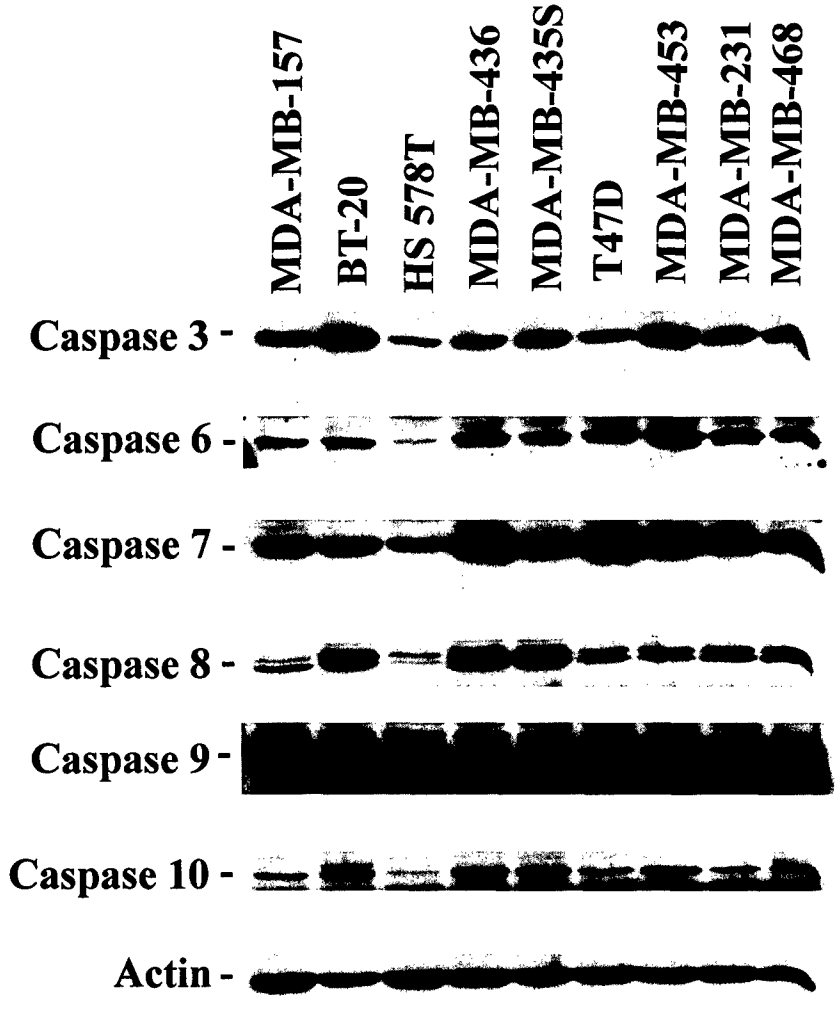



**Figure 1. Protein levels of caspases 3, 6, 7, 8, 9 and 10 in 9 breast cancer cell lines.**

Protein lysates were made from breast cancer cell lines. Individual caspases were probed with corresponding specific antibodies using Western blots. Actin levels are shown as loading controls.

Appendix B

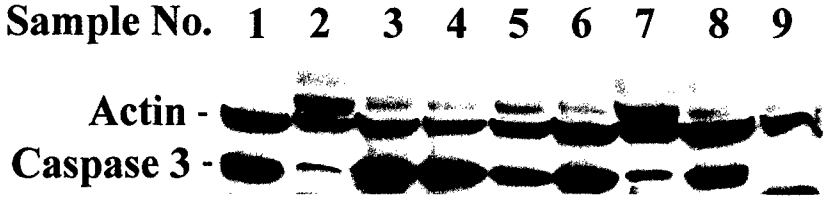
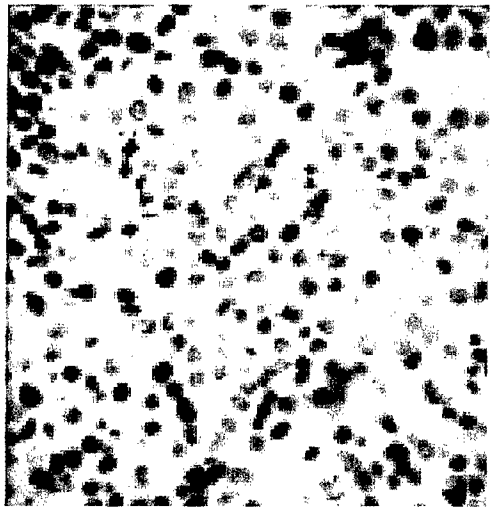


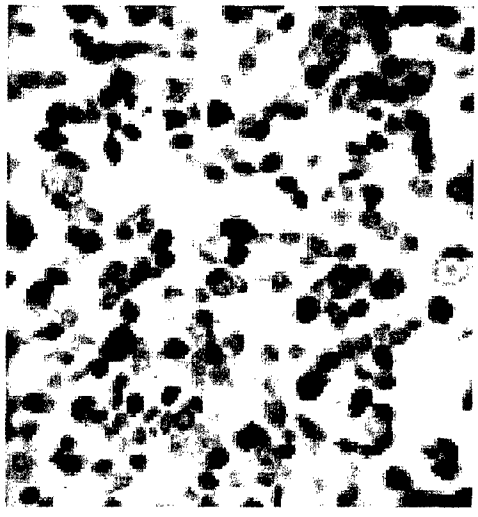

**Figure 2. Caspase 3 levels in snap-frozen breast cancer tissues.** Protein lysates were made from snap-frozen breast cancer tissues and separated with SDS-PAGE gel. Caspase 3 levels were detected using Western blot. Actin levels are shown as loading controls.

**Appendix B**

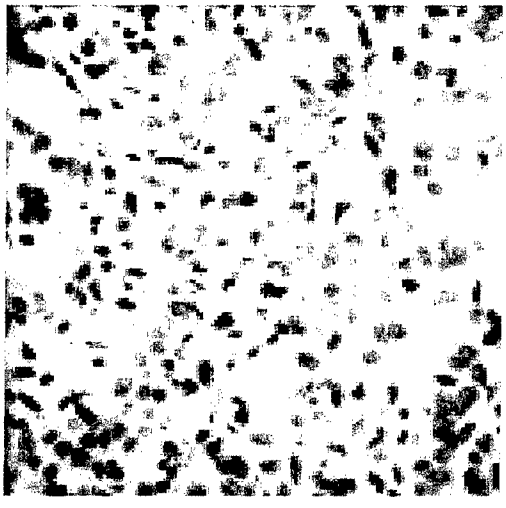
**A. MCF-7/pv**



**B. MCF-7/c3**



**C. Breast Cancer: Caspase 3 negative**



**D. Breast Cancer: Caspase 3 positive**

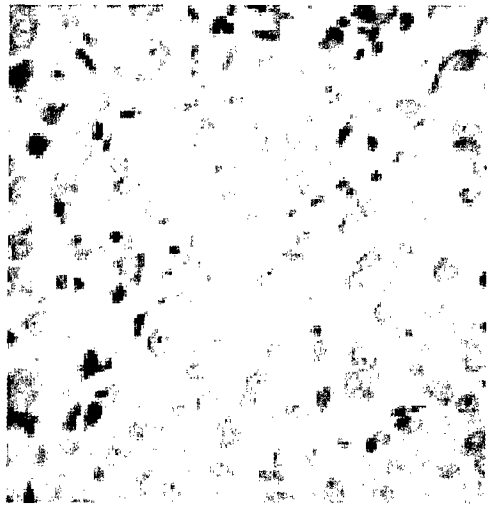


Figure 3. Detection of caspase 3 expression in breast cancer tissues by immunohistochemistry. A and B, control (MCF-7/pv) and caspase 3 reconstituted (MCF-7/c3) MCF-7 cells serve as negative and positive controls, respectively. C and D represent caspase 3 negative and positive breast cancer tissues, respectively.

## Appendix C

# Reconstitution of Caspase 3 Sensitizes MCF-7 Breast Cancer Cells to Doxorubicin Induced Apoptosis[1]

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**Running title**: Caspase 3 reconstitution mediated sensitization to doxorubicin

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## ABSTRACT

MCF-7, a breast cancer derived cell line, is deficient of caspase 3 and relatively insensitive to many chemotherapeutic agents. To study the association of caspase 3 deficiency and chemotherapeutic resistance, we reconstituted caspase 3 in MCF-7 cells and characterized their apoptotic response to doxorubicin. Western blots demonstrated that caspase 3 was constitutively expressed in the reconstituted MCF-7 cells. Both morphological observation and survival assays showed that caspase 3 reconstitution significantly sensitized MCF-7 cells to doxorubicin. Remarkably increased activation of caspases 3, 6 and 7, and cleavage of cellular death substrates were detected in the reconstituted MCF-7 cells post-doxorubicin. Our results demonstrated a specific role for caspase 3 in caspases 6 and 7 activation, and suggested a correlation between caspase 3 activity and dose-intensive effects of doxorubicin.

**Key words: Caspase 3; Apoptosis; Doxorubicin; Chemotherapy; Sensitization**

## INTRODUCTION

Chemotherapeutic resistance is a major problem in human oncology. Mechanisms of chemotherapeutic resistance are diverse and poorly defined for most cancer subtypes. Recent studies suggest that aberrant apoptosis (programmed cell death) likely contributes to this process (1). Apoptosis is a genetically controlled process that can be triggered by different extracellular and intracellular stimuli (2). Apoptotic execution requires coordinated activation of a special group of proteases, named caspases (3, 4). The activation of caspases is a signaling cascade mediated by proteolysis (5). Activated caspases subsequently cleave cellular death substrates and cause biochemical and morphological changes, leading to apoptosis (6). Fourteen mammalian caspases have been cloned (3, 7). Caspases 2, 8, 9 and 10 (apical caspases) initiate apoptosis and activate downstream caspases. Caspases 3, 6 and 7 (effector caspases) are activated by apical caspases and further cleave cellular death substrates (3).

Caspase 3 (also known as cpp32, yama and apopain) is a key caspase in this signaling cascade (8-12). Caspase 3 activity has been detected in apoptosis induced by a variety of apoptotic signals, including death receptor activation (13), growth factor deprivation (14), ionizing radiation (15), and treatment with granzyme B (16) or different chemotherapeutic agents (17). Caspase 3 knockout mice displayed abnormal brain tissue development due to lack of apoptosis (18). A growing number of substrates cleaved by caspase 3 have been identified, such as poly (ADP-ribosome) polymerase (PARP) (10), sterol-regulatory element-binding protein (SREBPs)(19), gelsolin (20), the U1-associated 70 kDa protein (21), D4-GDI (22), DNA fragmentation factor (DFF) (23), DNA dependent protein kinase $\delta$ and $\theta$ (24, 25), $\alpha$-fordrin (26) and huntingtin (27). Caspase 3 is believed to play a pivotal role in apoptotic execution.

Alterations in apoptosis associated genes are often observed in cancers. The p53 tumor suppressor gene, a key regulator in DNA damage induced apoptosis, is frequently mutated in human tumors (28). Overexpression of apoptotic inhibitors, such as bcl-2 and bcl-xL (29, 30), and down-regulated apoptotic promoting factors, such as Bax-α and Fas (31, 32), have been detected in primary tumors and tumor cell lines. These alterations have been linked to chemotherapeutic resistance (31, 33). Correction of these alterations has resulted in sensitization of the defective cells to chemotherapeutic agents (34).

Caspase 3 deficiency was recently detected in MCF-7 breast cancer cells. It is due to a deletion mutation in exon 3 of the gene (35). Given the important role of caspase 3 in apoptotic execution and the correlation between the alterations of other apoptotic regulators and chemotherapeutic resistance, we postulated that caspase 3 deficiency might also significantly contribute to chemotherapeutic resistance. Although caspase 3-like activity has been detected in the apoptosis induced by various chemotherapeutic drugs (17), the specific role of caspase 3 in this process warrants further investigation due to the overlapping activities among effector caspases (18, 36). To evaluate the role of caspase 3 in doxorubicin-induced apoptosis, we reconstituted caspase 3 in MCF-7 cells and characterized their apoptotic responses to the drug in comparison to the control cells. We found that reconstitution of caspase 3 significantly sensitized MCF-7 to doxorubicin-induced apoptosis.

## MATERIALS AND METHODS

### Cell culture, plasmid construction and transfection

MCF-7 cells were maintained in Iscove's Modified Dulbecco's Medium (IMDM) (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin. The pBabepuro/caspase 3 plasmid was constructed by treating BamH1/Pst1 caspase 3 cDNA insert from pBS/caspase 3 plasmid (a gift from Drs. Boothman and Pink) with T4 DNA polymerase and then subcloning into the blunt-ended pBabe/puromycin retroviral vector (37). MCF-7 cells were inoculated into 60 mm dishes at $3x10^5$ cells/dish and allowed to grow overnight. Two μg of caspase 3 encoding pBabepuro plasmid was mixed with 10 μl of Lipofectamin (Life Technologies, Gaitherburg, MD) and transfected into the cells according to the manufacture's instructions. Empty pBabepuro vector was also transfected as the control. Twenty-four hours after transfection, the cells were trypsinized, diluted, and inoculated into 96 well plates. Transfected cells were then selected with 2 μg/ml puromycin. Individual puromycin-resistant clones were screened for caspase 3 by Western blot. Five caspase 3 positive clones were pooled for further characterization. Morphological changes were observed and photographed with a phase contrast microscope.

### Drug treatment and sample collection

For doxorubicin (Bedford Labs, Bedford, OH) treatment in studies other than MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Thazolyl blue) assay (see below), 1 x $10^6$ cells were seeded into 60 mm dishes 24 hours before drug treatment. Various doses were

added to the dishes 18 hours before cell collection. Treated cells to be analyzed by flow cytometry were trypsinized. Cells to be analyzed by DEVD (Asp-Glu-Val-Asp) cleavage and Western blot were scrapped off the dish. In all cases, medium from individual dish, which might contain floating dead cells, was collected and mixed with the cell pellet from the same dish.

MTT survival assay

Three hundred cells were inoculated into each well of 96 well plates. Twenty-four hours later, the medium was replaced with new medium containing defined dose of doxorubicin. Six days after treatment, the medium was changed with phenol red free medium containing 50 μg/ml MTT (Sigma, St. Louis, MO). Three hours after incubation, MTT containing medium was removed. The incorporated dye was dissolved in 100 μl/well of dimethyl sulfoxide, and the plates were read at the wavelength of 570 nm using an ELISA reader. Eight parallel samples were treated in each group.

DEVD cleavage assay

Doxorubicin treated cells were washed with phosphate-buffered saline (PBS) and resuspended in lysis buffer (50 mM pH 8.0 Tris-HCl, 130 mM KCl, 1mM EDTA, 10 mM EGTA and 10 μM digitonin) at 320 μl/60 mm dish. Following incubation at 37° C for 10 minutes, the samples were spun for 3 minutes (5000 rpm), and the supernatant was collected. After adding 100 μl lysate to each well of a fluorometer plate, 100 μl of substrate solution, 2 μM DEVD-AMC (PharMingen, San Diego, CA) in lysis buffer, was added right before the reading. Fluorescence was measured in a microplate fluorometer (Cambridge Technology, Cambridge,

MA) using an exciting wavelength of 360 nm and emission wavelength of 460 nm. Results are reported as the fluorogenic activity over one hour ($T_{60}$-$T_0$). Samples were prepared in triplicate.

Western blot

PBS washed cells were treated with lysis buffer (38) on ice for 30 minutes. Lysed cells were centrifuged at 14,000 rpm for 10 minutes to remove cellular debris. Protein concentrations of the supernatant were determined using BCA™ Protein Assay (Pierce, Rockford, IL). Fifty μg of cell lysate was loaded onto each lane of a gel. Protein was separated by either 10% or 15% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked with 5% milk in tris-buffered saline-tween 20 (TBS-T) washing buffer (38) and probed with specific primary antibodies. Concentrations of the primary antibodies used were 1:500 to 1:2000 dilution. Antibodies against caspases 3 and 7 were purchased from Transduction Laboratories (Lexington, KY). Antibodies against caspase 6 and DFF were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The anti-lamin B antibody was from PharMingen (San Diego, CA). The anti-PARP antibody was from Boehringer Mannhem (Indianapolis, IN). Washed membranes were then probed with horse radish peroxide labeled anti-mouse, anti-rabbit, or anti-goat secondary antibodies (Amersham-Phamacia, Arlington Height, IL), respectively. The membranes were washed again and treated with Enhanced Chemiluminescence (ECL) reagents (Amersham-Phamacia, Arlington Height, IL). The specific protein bands were visualized by autoradiography (38).

Flow cytometry

Doxorubicin treated cells were trypsinized and washed with PBS. The cells were then fixed in 50 μl of 0.125% paraformaldehyde in PBS at 37° C for 5 minutes, followed by the addition of 450 μl of ice-cold methanol to each sample. After being washed three times with PBS containing 0.1% Triton X-100 and treated with RNase A (0.04 Kunitz units) for 30 minutes, the cells were then stained with 50 μg/ml of propidium iodide. Cell analysis was performed using a Coulter Epics 751 flow cytometer. The fraction of the total cell population present in each of G1, S, G2/M and the hypodiploid peak was obtained from DNA histograms by mathematical modeling using MPLUS software (38).

**RESULTS**

Stable reconstitution of caspase 3 in MCF-7 breast cancer cells

To reconstitute caspase 3, MCF-7 cells were transfected with pBabe/puro retroviral vector plasmid encoding a full-length pro-caspase 3 cDNA or empty vector as control. After puromycin selection, MCF-7 cell lines reconstituted with caspase 3 (MCF-7/c3), and control cells tranfected with pBabe/puro vector (MCF7/pv) were obtained. As shown in Fig.1, the protein levels of caspase 3 in MCF-7/pv, MCF-7/c3 and Jurkat cell controls were detected by Western blot. The caspase 3 specific antibody detected a strong protein band with a molecular weight of about 32 kDa in MCF-7/c3 and Jurkat cells but not in MCF-7/pv cells. Caspase 3 levels reconstituted in MCF-7/c3 cells were comparable to those in Jurkat cells that express high levels of caspase 3. The results below demonstrate that the reconstituted caspase 3 was

functional. We have since maintained MCF-7/c3 cells in culture for over one year, with stability in the expression of high levels of caspase 3.

Reconstitution of caspase 3 sensitizes MCF-7 cells to doxorubicin mediated killing.

To compare the sensitivity of MCF-7/c3 cells and MCF-7/pv cells to doxorubicin, we studied the viability and morphological changes of treated cells. After 6 days continuous exposure to doxorubicin (at concentrations of 0.064 to 200 nM), MCF-7/c3 cells were more sensitive than MCF-7/pv cells to doxorubicin, as detected by MTT survival assays (Fig. 2A). The survival fractions of MCF-7/c3 cells were significantly lower than that of MCF-7/pv cells in a dose dependent manner, indicating that caspase 3 reconstitution sensitized MCF-7 to doxorubicin treatment. Similar sensitization was also seen in the MCF-7/c3 cells treated with etoposide and taxol (data not shown).

Morphological changes commensurate with striking cytopathic differences in chemotherapeutic sensitization were observed in the caspase 3 reconstituted cells. When MCF-7/pv and MCF-7/c3 cells were treated with doxorubicin at concentrations of 0, 2.5, 5 and 10 μM for 18 hours, the differences between the two cell lines was evident at all doses (even the 2.5 μM group). This effect was magnified at increased concentrations. Cellular alterations included shrinkage, rounding, detachment, membrane blebbing, and segregation of cellular structure. At a concentration of 10 μM, MCF-7/c3 cells displayed diffused apoptosis as compared to MCF-7/pv cells, which showed only sporadic islands of cell death (Fig. 2B).

Activation of effector caspases in caspase 3 reconstituted cells

To verify that the above-described sensitization to doxorubicin was through caspase 3 mediated apoptosis, we analyzed the activation of effector caspases in MCF-7/c3 and MCF-7/pv cells using DEVD cleavage assay and Western blot. DEVD cleavage assay is a quantitative method that detects caspase 3-like activity (36). DEVD cleavage activity in doxorubicin treated MCF-7/pv cells was very limited, even in the cells treated with 5 μM doxorubicin. However, DEVD cleavage activity in MCF-7/c3 cells increased over 20-fold when the cells were treated with 2.5 μM of doxorubicin (Fig. 3A). Strong caspase 3 like activity in doxorubicin treated MCF-7/c3 cells suggests the role of caspase 3 reconstitution in the sensitization.

To detect the activation of specific effector caspases (indicated by sub-unit generation), Western blot was performed. After 18 hours treatment, activation of caspase 3 occurred with low dose doxorubicin (2 μM) and increased significantly at 10 and 50 μM (Fig. 3B). In contrast to minimal processing of caspase 7 in the treated MCF-7/pv cells, activation of caspase 7 in MCF-7/c3 cells was remarkably increased when the cells were treated with 10 or 50 μM doxorubicin (Fig. 3C). The result indicated that caspase 7 activation in doxorubicin treated cells was caspase 3 dependent. This observation is consistent with our reported finding of granzyme B induced apoptosis (16).

Analysis of caspase 6 activation in the two cell lines revealed more specific action of caspase 3 (Fig. 3C). As indicated by the p32 band product in MCF-7/pv cells, caspase 6 activation occurred at low levels in the absence of caspase 3. Reconstitution of caspase 3 significantly enhanced caspase 6 activation at 10 and 50 μM of doxorubicin. Since the combined size of pLarge and pSmall sub-units of caspase 6 is about 32 kDa (39), the appearance of p32 band in MCF-7/pv cells suggests that caspase 6 was processed by a caspase, other than caspase 3, between the pro-peptide and pLarge sub-unit. The disappearance of the p32 band and an

increase in the pLarge sub-unit (p20) in treated MCF-7/c3 cells suggests that caspase 3 processes caspase 6 between the pLarge and the pSmall sub-units. It further suggests that efficient activation of caspase 6 requires two steps, with caspase 3 dependent activation being one of them. These results demonstrate that MCF-7/c3 cells had remarkably increased effector caspase activity in response to doxorubicin.

Cleavage of cellular death substrate in MCF-7/c3 cells

Since proteolytic cleavage of cellular death substrates by activated caspases is responsible for cellular dysfunction and structural destruction (6), we studied the cleavage of PARP, lamin B, and DFF as representative substrates in control and the reconstituted cells. As shown in Fig. 4, there was only limited cleavage of all three substrates in MCF-7/pv cells (even if the doxorubicin concentration was as high as 50 μM). In contrast, all three substrates were almost completely cleaved in MCF-7/c3 cells treated with 10 or 50 μM doxorubicin. Since increased caspase activities were seen under these conditions (Fig. 3B and 3C), the observed proteolysis was a consequence of caspase 3 reconstitution.

Caspase 3 was required for doxorubicin induced DNA fragmentation

DNA fragmentation is a key feature associated with apoptosis (6). Caspase 3 is required for DNA fragmentation in tumor necrosis factor-α (TNF-α) induced apoptosis (35). To examine the effect of caspase 3 reconstitution on DNA fragmentation in doxorubicin induced apoptosis, we analyzed the DNA fragmentation by flow cytometry in doxorubicin treated MCF-7/c3 and MCF-7/pv cells. As shown in Fig. 5, significant DNA fragmentation (the hypodiploid peak) was only detected in doxorubicin treated MCF-7/c3 cells. Agarose gel analysis showed that DNA

ladders were only present in doxorubicin treated MCF-7/c3 cells (data not shown). This suggests that caspase 3 was also required for DNA fragmentation in doxorubicin induced apoptosis.

## DISCUSSION

In this report we described the establishment of a stable MCF-7 cell line reconstituted with caspase 3. This line was useful for studying the specific role of caspase 3 and caspase 3 dependent signaling in response to doxorubicin. As demonstrated by MTT assay and morphologic data, caspase 3 reconstitution sensitized MCF-7 cells to doxorubicin induced apoptosis. Increased DEVD cleavage and amplified activation of caspases 6 and 7 were also observed in caspase 3 reconstituted cells. Significant increases in the proteolysis of cell death substrates further verified caspase 3 mediated sensitization in doxorubicin induced apoptosis. We have found that caspase 3 reconstitution also rendered MCF-7 cells more sensitive to etoposide and taxol in a similar fashion (data not shown).

Doxorubicin, which is one of the most active chemotherapeutic agents used in clinical oncology, can trigger apoptosis through several mechanisms. As with most chemotherapeutic agents, it induces DNA damage by interacting with topoisomerase II, leading to DNA breakage (40). It can also induce membrane alterations and the generation of ceramide at higher concentrations (41). Recently it has been reported that up-regulation of the Fas/Fas-ligand system is also involved in doxorubicin mediated killing (42). Although we did not investigate the initiation pathways in the present study, our results show that caspase 3 plays a critical role in the downstream events of doxorubicin mediated killing. Reconstitution of caspase 3 restores the integrity of doxorubicin induced killing mechanisms.

When MCF-7/c3 cells were treated with doxorubicin for 18 hours, caspase activation and death substrate cleavage displayed a sharp increase when the drug concentration changed from 2 μM to 10 μM (Fig. 3B, 3C and 4). This suggests that there may be a concentration threshold for doxorubicin to induce maximal caspase 3 mediated apoptosis. This is consistent with the clinical benefit observed with dose-intensive doxorubicin (e.g. in node positive breast cancer patients) (43). The potential correlation between caspase 3 activation and the dose-intensive effect of doxorubicin is being investigated. Although stronger sensitization was observed in the groups treated with high dose of doxorubicin, caspase 3-mediated sensitization was also observed when the doxorubicin concentration was lower. As indicated by our MTT results (Fig. 2A), caspase 3-mediated sensitization still occurred at lower doxorubicin concentrations (0.064 - 200 nM) with prolonged exposure (6 days). The DEVD cleavage assay, which is more sensitive than Western blot, showed a striking difference between the two cell lines when treated with 2.5 uM doxorubicin for 18 hours. As proposed by Han et al (44), we also think that doxorubicin may induce two types of cellular responses—slow cell death at low concentration and rapid cell death at high concentration. This may be due to more mechanisms activated at a higher doxorubicin concentrations.

Although involvement of caspase 3 activation in chemotherapy induced apoptosis has been reported by many groups (17, 45-48), little work has been done in dissecting the specific roles of individual effector caspase in this process. In our experiments, comparison between caspase 3 deficient and reconstituted cell lines more specifically defined the specific role of caspase 3 in doxorubicin-induced apoptosis and in the activation of other effector caspases. Although caspase 3, 6 and 7 are all categorized as effector caspases (3, 5), our results demonstrated an additional apical-like nature of caspase 3. These results were the direct *in vivo*

evidence showing that activation of caspase 6, and especially caspase 7, were largely dependent on caspase 3 activation (Fig. 3B and 3C). Although caspase 6 activation was detected in caspase 3 deficient cells, efficient maturation of caspase 6 required caspase 3 processing between the pLarge and pSmall sub-units (Fig. 3C). By direct cleavage and amplification through the activation of other effector caspases, caspase 3 reconstitution resulted in a striking increase in death substrate cleavage and DNA fragmentation (Fig. 4 and 5). These results support the essential role of caspase 3 in doxorubicin induced apoptosis.

Distinct differences in apoptotic activities between MCF-7/pv and MCF-7/c3 cell lines in response to doxorubicin treatment underscore the possible significance of caspase 3 in cancer resistance. Caspase 3 reconstitution sensitized MCF-7 breast cancer derived cells to a commonly applied chemotherapeutic agent, suggesting that caspase 3 deficiency may contribute to chemotherapeutic resistance. With better definition of caspase expression/mutation in clinical breast cancer specimens, caspase alterations may be linked to poorer prognosis and chemotherapeutic resistance. We have also found that caspase 3 reconstitution sensitized MCF-7 cells to radiotherapy, granzyme B, and TNF-$\alpha$ induced apoptosis (16, and unpublished data). Therefore, it appears that caspase 3 deficiency may have a broader impact including both therapeutic resistance and immune raised anti-tumor mechanism. Our preliminary results show down-regulation or deficiency of caspase 3 in many breast cancer specimens (unpublished data). Caspase deficiencies have also been identified by others in non-breast cancer specimens as well (49).

## ACKNOWLEDGEMENTS

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## LEGENDS

Fig. 1. Reconstitution of caspase 3 in MCF-7 cells. Protein levels of caspase 3 in Jurkat, MCF-7/pv and MCF-c3 cells were detected suing Western blot. MCF-7/pv and MCF-7/c3 cells were MCF-7 cell transfected with pBabe/puro vector and the vector encoding caspase 3 cDNA, respectively.

Fig. 2. Enhanced killing in MCF-7/c3 cells treated with doxorubicin. A. MTT assay. Doxorubicin was added to MCF-7/pv and MCF-7/c3 cells in 96 well plates 24 hours after inoculation and left for 6 days before viability was determined using MTT assay. B. Morphology observation. MCF-7/pv (Fig. 2Ba and 2Bb) and MCF-7/c3 (Fig. 2Bc and 2Bd) were treated (Fig. 2Bb and 2Bd) with 10 μM doxorubicin for 18 hours, as compared with untreated (Fig. 2Ba and 2Bc) cells. Photographs were taken under a phase contrast microscope (10 x 20).

Fig. 3. Effect of caspase 3 reconstitution on effector caspase activation in MCF-7/pv and MCF-7/c3 cells. A. DEVD cleavage activity. MCF-7/pv and MCF-7/c3 cells were treated with doxorubicin at indicated concentrations for 18 hours before the lysate was prepared for fluorogenic assay. B. Activation of caspase 3 in MCF-7/c3 cells. C. Activation of caspases 6 and 7 in MCF-7/pv and MCF-7/c3 cells. For B and C, the cells were treated with doxorubicin at the indicated concentration for 18 hours before the lysate was prepared for Western blot. 50 μg of lysate protein was separated with SDS-PAGE gel. The caspases were probed with specific antibodies against caspase 3, 6 and 7, respectively.

Fig. 4. Proteolytic cleavage of PARP, lamin B and DFF. The conditions for sample preparation and Western blot were the same as in Fig. 3 B and C. Cleavage of PARP, lamin B and DFF was detected with corresponding specific antibody respectively.

Fig. 5. Flow cytometry analysis of DNA fragmentation. MCF-7/pv and MCF-7/c3 cells were treated with doxorubicin at indicated concentrations for 18 hours. The cells were collected, fixed and stained with propidium iodide. DNA content was analyzed using flow cytometry.

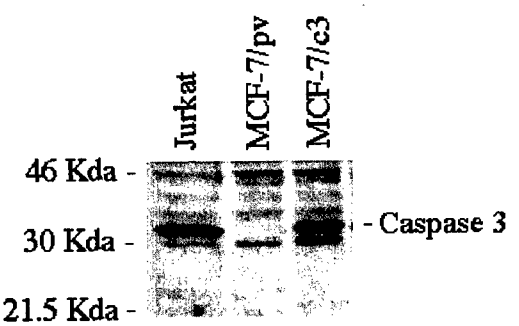


Figure 1

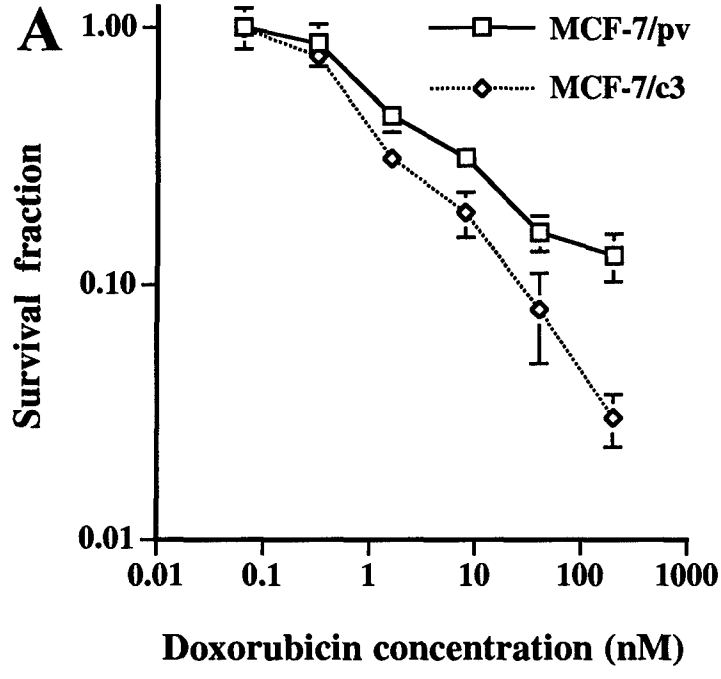
A
1.00
0.10
0.01
Survival fraction
MCF-7/pv
MCF-7/c3
0.01
0.1
1
10
100
1000
Doxorubicin concentration (nM)

Figure 2A

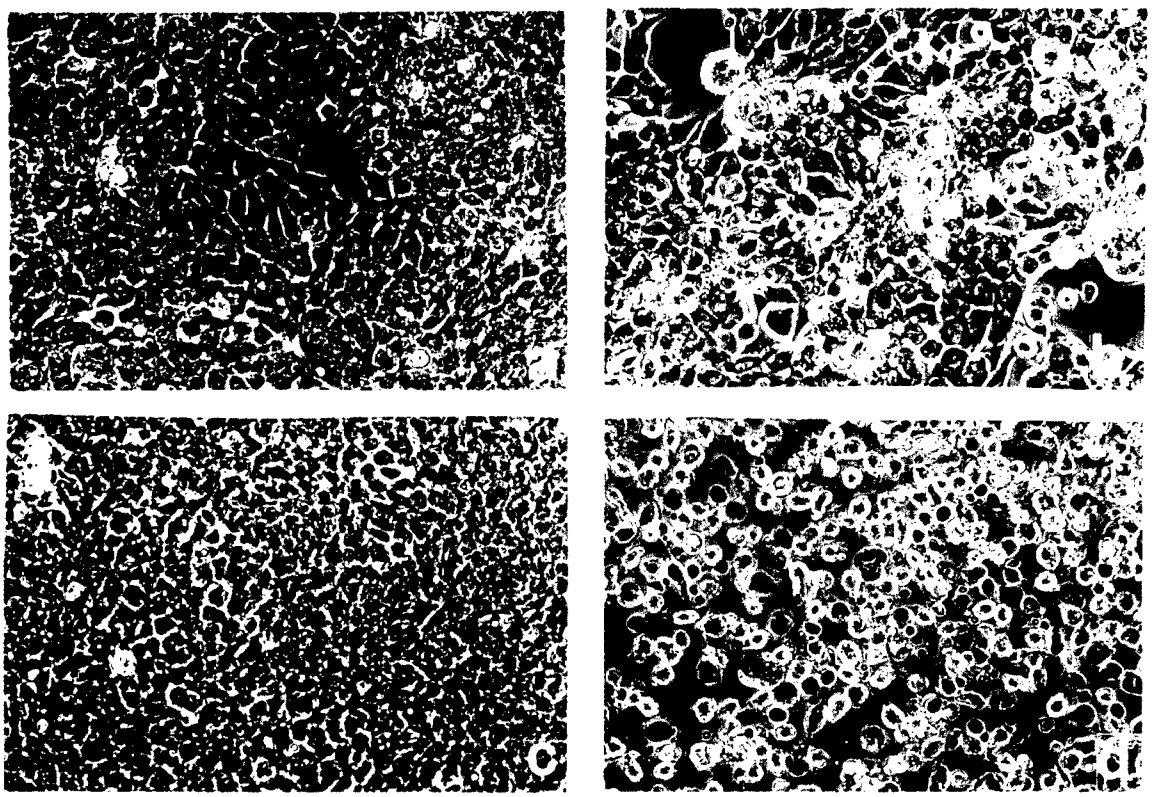

Figure 2B

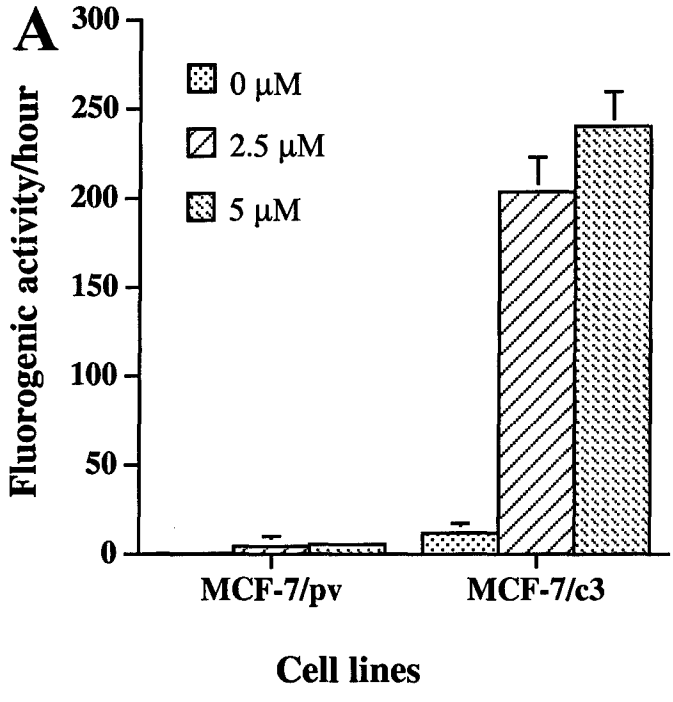
A
300
250
200
150
100
50
0
Fluorogenic activity/hour
0 μM
2.5 μM
5 μM
MCF-7/pv
MCF-7/c3
Cell lines

Figure 3A

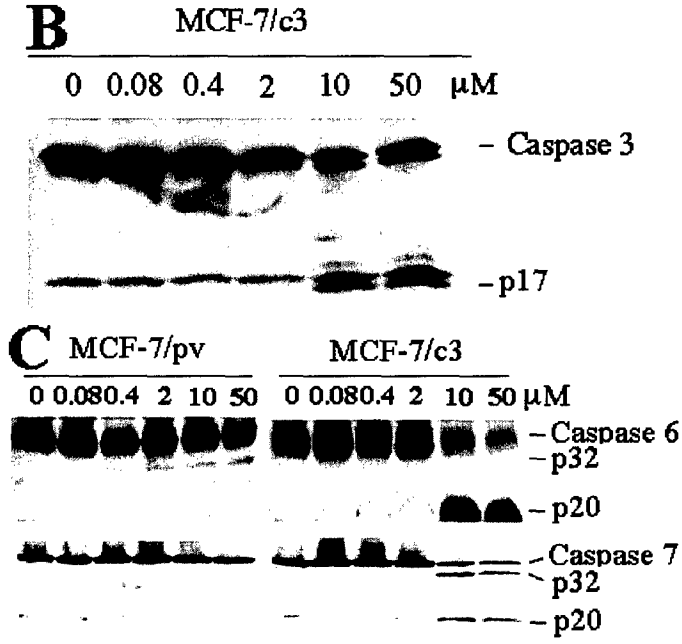
B
MCF-7/c3
0 0.08 0.4 2 10 50 μM
– Caspase 3
– p17
C
MCF-7/pv
MCF-7/c3
0 0.080.4 2 10 50
0 0.080.4 2 10 50 μM
– Caspase 6
– p32
– p20
– Caspase 7
– p32
– p20

Figure 3B

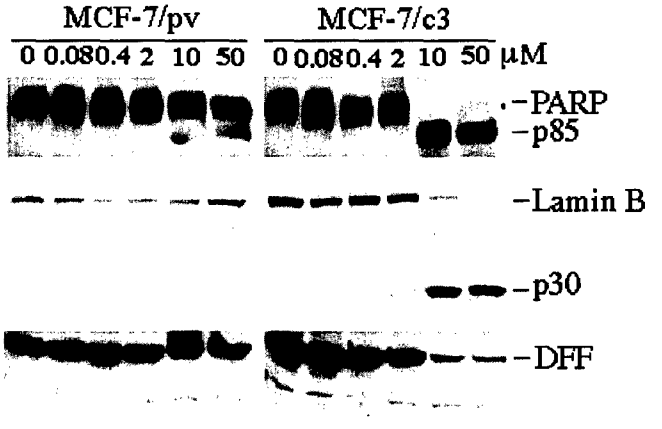
MCF-7/pv
MCF-7/c3
0 0.08 0.4 2 10 50 0 0.08 0.4 2 10 50 μM
-PARP
-p85
-Lamin B
-p30
-DFF

Figure 4

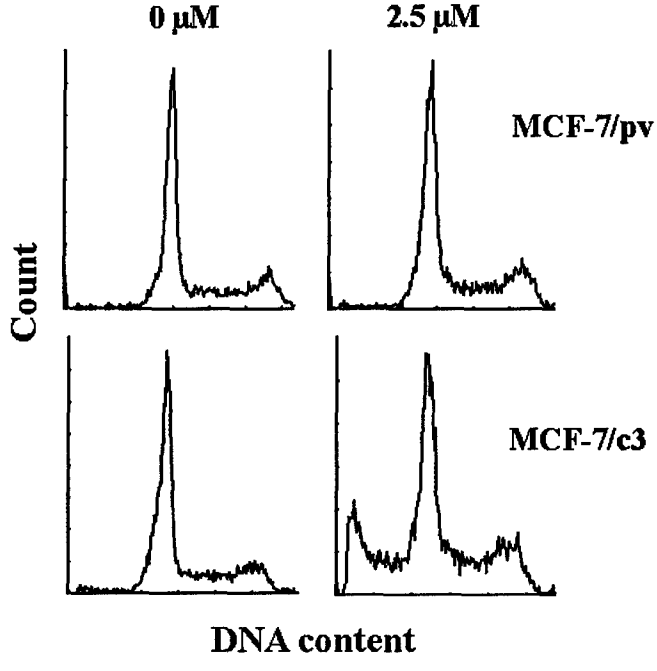


Figure 5

**Appendix D**: Abstract presented at the 22nd San Antonio Breast Cancer Symposium (1999)

## Reconstitution of caspase 3 sensitizes MCF-7 breast cancer cells to chemotherapeutic agent induced apoptosis

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Apoptosis plays important roles in cellular development and homeostasis. Aberrant apoptosis has been associated with the carcinogenesis and therapeutic resistance of cancer. Although apoptosis can be triggered by different stimuli, the involved signaling pathways ultimately converge to activate a group of proteases, called caspases. MCF-7 breast cancer cells, which are resistant to many apoptotic stimuli, have been found to be caspase 3 deficient. To study the correlation between caspase 3 deficiency and chemotherapy resistance, we reconstituted caspase 3 in MCF-7 cells and have now characterized their response to several chemotherapeutic agents. Caspase 3 was expressed in MCF-7 cells by transfecting the cells with pBabe/puro retroviral vector encoding caspase 3 cDNA. Constitutive expression of caspase 3 was demonstrated by Western blots. Caspase 3 expressing MCF-7 cells and the cells transfected with a control vector were treated with doxorubicin. Flow cytometry and MTT assays showed that caspase 3 expression rendered MCF-7 cells significantly more susceptible to doxorubicin, which induced cell killing via apoptosis. Decreased survival fraction was detected in caspase 3 expressing cells as compared to control cells. Apoptosis medicated by the reconstituted caspase 3 was demonstrated by increased DEVD cleavage activities, activation of effector caspases and cleavage of cellular death substrates. These results demonstrate the specific role of caspase 3 in chemotherapy induced apoptosis, suggesting that MCF-7 cell caspase 3 deficiency might contribute to chemotherapeutic resistance. The results also suggest that genetic reconstitution of caspase 3 might enhance chemotherapy sensitivity in breast cancers.

**Appendix E:** Abstract presented at the 91st America Association of Cancer Research (AACR) Annual Meeting

## Reconstitution of caspase 3 sensitizes MCF-7 cells to radiation induced apoptosis

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Appropriate regulation of apoptosis is required for effective tumor killing in different anti-tumor therapies, including radiation treatment. Abnormal expression of apoptotic regulators has been associated with resistance to radiation induced apoptosis. Caspase 3, which is a key factor in apoptosis execution, is deficient in MCF-7 breast cancer cells. To study the correlation between caspase 3 deficiency and radio-therapeutic resistance, we characterized the apoptosis of control and caspase 3 reconstituted MCF-7 cells treated with ionizing radiation. Survival assays showed that caspase 3 reconstitution significantly sensitized MCF-7 cells to radiation mediated killing. Enhanced apoptosis in caspase 3 reconstituted cells was indicated by increased DEVD cleavage activity and the activation of caspases 6 and 7. Consistently, cleavage of cellular death substrates, such as PARP and lamin B, increased significantly in caspase 3 reconstituted cells. In comparison to caspase 3-reconstituted MCF-7 cells treated with doxorubicin and TNF-□, cleavage of actin was more obvious in irradiated MCF-7 cell reconstituted with caspase 3 (as adjusted by PARP and lamin B cleavage), suggesting the subtle difference between radiation and chemotherapy induced activation of caspase cascade. These results demonstrate the pivotal role of caspase 3 in radiation induced apoptosis and suggest that caspase 3 deficiency might contribute to radioresistance in clinical treatment.